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CULTIVATION OF THE GONOCOCCUS *

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The gonococcus was first cultivated by Bumm¹ on coagulated human placental serum in 1885, six years after its discovery by Neisser.² Wertheim³ used a peptone agar with serum as a medium; the serum was inoculated with the infected material and added to the fluid agar in the proportion of two to one. Wassermann⁴ used pig serum agar with glycerol and nutrose. Thalman⁵ cultivated the gonococcus on plain agar, using a medium which in reaction was between the neutral points of litmus and phenolphthalein. He emphasized the importance of the reaction of the medium. Vannod⁶ isolated and maintained several strains of gonococcus on plain agar. Gurd⁷ suggested a medium of nutrient agar with a small quantity of defibrinated sterile human blood. Martin⁸ published a review of methods of cultivating the gonococcus and of its differentiation from other gram-negative diplococci. He used beef infusion agar, the reaction of which was 0.6% acid to phenolphthalein, and moistened the surface with inactivated human serum. Hirshfelder⁹ published a new formula using bullock testicular infusion agar. Warden¹⁰ used a culture medium which contained ascitic or hydrocele fluid, blood or blood serum; later he used defibrinated rabbit blood with salt-free veal-peptone-agar, neutral to phenolphthalein, the medium being dried until the surface cracked. Vedder¹¹ described a starch agar, the reaction of which was 0.2 to 0.5% acid to phenolphthalein. Hall¹² made a testicular infusion agar. Thomson¹³ used agar with human blood plasma. The more recent contributions on the cultivation of the gonococcus deal chiefly with the oxygen tension requirements and the importance of hydrogen-ion concentration. Nowak, in 1908, and Wherry and Oliver¹⁴ reduced the oxygen tension for the cultivation of the gonococcus with a growth of *B. subtilis*. Chapin¹⁵ suggested the cultivation of the gonococcus in an atmosphere of CO₂. Ruediger¹⁶ felt that the exclusion of air was

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¹ Deutsch. med. Wchnschr., 1885, 53, p. 910.

² Centralbl. f. d. med. Wissensch., 1879, 17, p. 497.

³ Deutsch. med. Wchnschr., 1891, 17, p. 1351.

⁴ Berl. klin. Wchnschr., 1897, 34, p. 685.

⁵ Centralbl. f. Bakteriologie, I, O., 1900, 27, p. 828.

⁶ Centralbl. f. Bakteriologie, I, O., 1905, 40, p. 62.

⁷ Jour. Med. Research, 1908, 18, p. 291.

⁸ Jour. Path. & Bacteriol., 1910-11, 15, p. 76.

⁹ Jour. Am. Med. Assn., 1914, 62, p. 776.

¹⁰ Jour. Infect. Dis., 1913, 12, p. 93.

¹¹ Jour. Infect. Dis., 1915, 16, p. 385.

¹² Jour. Bacteriol., 1916, 1, p. 343.

¹³ Brit. Med. Jour., 1917, 1, p. 869.

¹⁴ Jour. Infect. Dis., 1916, 19, p. 288.

¹⁵ Ibid., 23, p. 342.

¹⁶ Ibid., 24, p. 376.

advantageous. Herrold¹⁷ reduced the oxygen tension on plates in conjunction with *B. subtilis*. Swartz¹⁸ described a simple method for reducing oxygen tension by heating inoculated tubes at the mouth by passing them over a flame to rarify the air within and then inserting a rubber stopper.

From this brief resume, it is evident that there is no uniformly satisfactory culture medium or method of cultivation of the gonococcus.

NOTES ON THE DIFFERENT KINDS OF CULTURE MEDIUMS

A comparison of various kinds of culture mediums that have been used for the isolation and cultivation of the gonococcus was made. The mediums tried were ascitic agar, blood agar, nutrient agar with blood serum, Vedder's¹¹ starch agar, Loeffler's blood serum, testicular agar, and Avery's¹⁹ oleate-hemoglobin culture medium. Testicular infusion agar made according to Hall's¹² formula gave the most luxuriant growth of stock cultures. To this was added substances, such as blood or serum, which would favor the growth of gonococci from clinical cases. The addition of from 0.5 to 2.5% human blood to testicular agar produces a very good growth and is well adapted for isolation purposes. Defibrinated rabbit blood in proportions of 1 to 5% may be used. Blood, either human or rabbit, is added to agar at 40 C. Unheated or red blood agar produces a slightly better growth than chocolate or heated blood agar.

Human blood serum placed on the surface of testicular agar increases the growth materially. There was no difference in the growth of the organisms whether the serum was unheated or heated for one half hour at 56 C. The mixing of blood serum with the agar in proportions of one to three gives no better results than when the serum is simply smeared on the surface.

Martin⁸ asserts that the value of blood serum and urine is not due to the addition of albuminous material but to substances such as phosphates, which act as buffers and equalize the reaction.

Vedder's starch agar, Avery's¹⁹ oleate-hemoglobin medium, gelatin and nutrient agar cannot be relied on for isolation purposes since some strains of the organism refuse to grow on these mediums.

The importance and value of each supposedly essential ingredient of culture mediums, as well as the effect of the addition of other substances, such as bile, starch, etc., were studied.

¹⁷ Jour. Am. Med. Assn., 1920, 74, p. 1716.

¹⁸ Jour. Urology, 1920, 4, p. 325.

¹⁹ Jour. Am. Med. Assn., 1918, 71, p. 2050.

The Effect of Glucose.—Ruediger¹⁶ thinks that the addition of glucose is unfavorable. Cole²⁰ says that 1% glucose has no effect on initial growth, but shortens the viability of the cultures.

We have found that the presence of 0.5% glucose will cause certain strains to present visible colonies earlier, and also to show a more luxuriant growth than when no glucose was used. With other strains there seems to be no appreciable difference.

The Value of Peptone.—The importance of peptone was shown by experiments with 6 strains of the gonococcus. It was found that the growth is better on medium containing from 1.5 to 3% peptone than when lesser amounts are used.

The Effect of Salt.—Warden¹⁰ states that physiologic salt solution and also distilled water favor autolysis. Martin⁸ says that the organism loses its vitality in normal salt solution. We have confirmed these statements. Gonococci suspended in normal salt solution (0.7%) and transferred to tubes of culture medium failed to grow. Whenever we wished to use suspensions of the organisms we used a testicular infusion of P_H 7.4 which contained 0.3% monobasic sodium phosphate, 0.5% of glucose and 2% peptone. The gonococcus can also be grown in this broth.

We used monobasic sodium phosphate in from 0.2 to 0.3% in place of sodium chloride in the preparation of all culture mediums for gonococci. It is essential to use chemically pure salt since others seem to vary considerably in composition and ability to ionize. On several occasions we experienced considerable difficulty with the reaction of culture mediums. When using 0.3% of the phosphate (as recommended by Hall) the reaction changed from P_H 7.4 to P_H 6.5 by autoclaving, although the routine method of preparation was used and great care was taken not to autoclave longer than 20 minutes at 15 pounds of pressure. When the amount of monobasic sodium phosphate was reduced to 0.2%, the reaction adjusted to P_H 7.8 was found after autoclaving to be P_H 7.4. We consider the reaction of the medium to be of great importance as even stock cultures which show considerable adaptability to changes of medium, reaction, etc., show inhibition of growth when the reaction does not come within the optimum range.

Temperature Requirements.—One of the distinctive characteristics of the gonococcus is its failure to grow at room temperature. The

²⁰ Jour. Path. & Bacteriol., 1916-17, 21, p. 267.

maximum growth of the gonococcus occurs at 37.5 C. Jenkins²¹ states that at a temperature of 35 C., in a moist atmosphere growth appears at the eighth to tenth hour, whereas in a dry atmosphere at 37.5 C. growth was not noticed until the sixteenth hour.

We have found that growths will occur at temperatures as low as 33 C., and as high as 39 C. The growths were, however, scanty and cultures kept at these temperatures lose their viability in a few days. A temperature of 42 C., maintained for several hours does not kill cultures—growth again taking place after the temperature is reduced to 37.5 C.

Herrold¹⁷ emphasizes the necessity of warming the plates in the incubator for one-half hour before inoculation as a measure for inducing a good growth of the gonococcus. We have found this practice desirable and have adopted it for routine work.

The Reaction of Culture Mediums.—The optimum reaction for the growth of the gonococcus on artificial culture mediums is from P_H 7.4 to 7.6. This was determined by a series of tests with several strains, on mediums ranging in reaction from P_H 5.4 to 8.0. The results are shown in table 1.

TABLE 1
INFLUENCE OF HYDROGEN-ION CONCENTRATION ON GROWTH OF GONOCOCCUS

Gono- coccus Strains	P _H 5.4	P _H 6.0	P _H 6.4	P _H 7.0	P _H 7.4	P _H 7.6	P _H 8.0
1	+	+	+	+	++	++	+
2	+	+	+	+	++	++	+
3	0	+	+	+	++	++	+
4	0	+	+	+	++	++	+
5	0	0	+	+	++	++	+
6	0	0	+	+	++	++	+

0 = no growth, + = slight growth, ++ = good growth.

As seen in table 1, certain strains failed to show any growth when the hydrogen-ion concentration was 6 or lower. All strains had been growing artificially for some time.

Cole²⁰ gives a P_H range for the gonococcus from 6.5 to 9.1 with the optimum at P_H 7.6. Swartz¹⁸ found that growth occurred within a range of P_H 6.6 to 8.6; he states that the reaction of the culture mediums is not an important factor in the growth of the gonococcus. Martin⁸ maintains that the reaction of culture mediums is of prime importance. He found that the best growths of the organism were obtained when the medium was 0.6% acid to phenolphthalein. He

²¹ Ibid., 1921, 24, p. 160.

also states that "degrees of reaction beyond the limits of neutrality to an 0.8% acidity to phenolphthalein are distinctly unfavorable."

The Importance of Moisture in Culture Mediums.—It is of great importance to have the medium of the right moisture content. On too dry a medium the growth is scant or absent. We have found that the proper amount of moisture can be insured by using 2.5% granular agar. Shredded agar varies somewhat in moisture content and composition and culture mediums in which it is used accordingly are not uniform in consistency and constituents.

The tubes are stored in a moist chamber and slanted just before use. If tubes are slanted immediately after sterilization, the cotton plugs should be replaced by corks and dipped in paraffin. These tubes contain a moderate amount of water of condensation. With the exception of Warden,¹⁰ all workers have seen the necessity of a moderately moist culture medium.

We have found it advantageous to keep a dish of water in the incubator at all times in order to insure proper moisture conditions.

Reduced Oxygen Tension.—It has been claimed by Wherry and Oliver,¹⁴ Herrold,¹⁷ Swartz,¹⁸ and others, that reduced oxygen tension is essential if a good growth of the gonococcus is to be obtained.

Herrold reduced the oxygen tension by means of the *B. subtilis*; Swartz reduced it by passing the mouth of the inoculated tube through a flame several times and maintaining the diminished oxygen tension by corking the tube tightly with a rubber stopper. Reduction of the amount of oxygen has also been obtained by replacing a portion of the air with carbon dioxide.

We have tried the several methods suggested but have not been able to secure better results than when the organisms were grown in the presence of a normal oxygen tension. It is probable that the more satisfactory results attained by certain investigators is due, not to a reduced oxygen tension, but to the presence of an increased amount of moisture due to the technic used to exclude air.²²

The Effect of Liver and Bile.—The addition of dry powdered liver to testicular agar did not improve the growth of the gonococcus. Culture mediums made from distilled water with 1 and 2% dry liver, peptone, glucose and monobasic sodium phosphate and the reaction adjusted to P_H 7.4, maintained the gonococcus for two subcultures. The third subcultures failed to grow.

²² Kolaman, E. F.: J. Bacteriol., 1919, 4, p. 571.

Fresh ox-bile added to testicular agar in from 0.3 to 0.5% inhibited the growth of several strains of gonococci and slightly improved the growth of several others. Dry ox-gall added to testicular agar in from 0.5 to 1.0% inhibited the growth of gonococci slightly but did not inhibit the growth of the two strains of strephylococci and one of the colon bacillus on which such was tried. In a 0.2% preparation it also failed to influence the growth of the gonococcus. Bile salts in the form of sodium taurocholate and sodium glycocholate were added to testicular agar in proportions of 3 to 5%. The growth of five strains of gonococci was slightly inhibited by this. No effect was noticed on a culture of the colon bacillus, nor on two strains of staphylococci whereas one strain of the latter was slightly inhibited.

Fresh Tissue as Vitamines.—The addition of fresh tissue such as the liver and kidney of guinea-pigs to the testicular agar did not augment the growth of the gonococcus.

The Effect of Sodium Oleate.—Sodium oleate in the proportion of 1:1,000 was added to testicular agar for the purpose of reducing surface tension. Fairly good growths were obtained with 4 strains of the gonococcus but growth appeared later than in the controls. One strain showed no growth in 48 hours. The growth of 3 strains of staphylococci on this medium was as good as that on the control tubes.

The Effect of Starches.—A comparison was made of the growth of the gonococcus on agars containing the following starches: arrow-root, barley, bean, buckwheat, corn, ginger, pea, potato, rye, rice, sago and tapioca. Starch added to testicular agar does not improve the growth. Vedder¹¹ used cornstarch. We have found that the use of potato starch will in some instances result in better growths than when corn or other starches are used.

PHENOL RED AS AN INDICATOR IN FERMENTATION REACTIONS OF THE GONOCOCCUS

The gonococcus is very sensitive to the reaction of the medium. To get the best results in any test which depends on the growth of the organism, optimum conditions for growth should be provided. In most of the work on fermentation reactions of the gonococcus, litmus has been used as the indicator. The color change of litmus is approximately at P_H 6.8, while the maximum growth of the gonococcus is obtained at P_H 7.4. Phenolsulphonephthalein or "phenol red" is an indicator which has its range at the optimum reaction for the gono-

coccus. Enough indicator was added to the medium to give a decided rose-red color. Phenol red does not have any inhibitory action on the gonococcus when used in testicular agar in concentrations decidedly higher than necessary to show the changes in the reaction of the medium caused by the growth of the gonococcus.

In most of the previous work on the fermentation of sugars by the gonococcus, liquid mediums with a sugar concentration of 1% was used. Growth in liquid medium is always slower than on solid. We used solid culture mediums with 0.5% sugar. Dextrose was the only sugar in which acid production was observed. Lactose, saccharose and levulose in 1% concentration did not show acidity in 48 hours.

The results produced with 0.5% dextrose differed somewhat from those with 1.0% of the sugar. Acid is produced in both. When 0.5% dextrose was used, acidity was present in 9 hours. There was no apparent change in reaction of the medium after it had become acid until about the twenty-second hour; at this time 4 out of 5 strains began to show a change from acidity to alkalinity. The reaction gradually changed to complete alkalinity at about the fifty-seventh hour when the medium was strongly alkaline in all cases except one. When 1.0% dextrose was used no secondary alkalinity was observed. Fermentation of 0.5% dextrose in testicular agar is shown in table 2.

TABLE 2
FERMENTATION OF 0.5% DEXTROSE IN TESTICULAR AGAR WITH A REACTION OF PH 7.4, AND
USING "PHENOL RED" AS AN INDICATOR

Strain	5 Hours	6 Hours	8 Hours	9 Hours	10 Hours	12 Hours	22 Hours	23 Hours	24 Hours	32 Hours	57 Hours	64 Hours
1	+	+	A	A	A	A	A+alk.	A+alk.	A+alk.	A+alk.	Alk.	Alk.
2	+	+	+	A	A	A	A	A	A	A	A+alk.	A+alk.
3	+	+	A	A	A	A	A+alk.	A+alk.	A+alk.	A+alk.	Alk.	Alk.
4	+	+	+	A	A	A	A+alk.	A+alk.	A+alk.	A+alk.	Alk.	Alk.
5	+	A	A	A	A	A	A+alk.	A+alk.	A+alk.	A+alk.	Alk.	Alk.

+ = Growth—no change in reaction of medium.

A = Acidity of medium.

A + alk. = Acid medium, showing beginning secondary alkalinity.

Alk. = Secondary alkalinity complete.

The reason for this difference in acid and alkali formation probably lies in the fact that the 1% dextrose medium contains more dextrose than can be utilized by one culture of the organism, whereas in the 0.5% medium the dextrose is used up before the culture reaches the end of its growth. In all probability, if we could keep a given culture growing for a long period of time on a 1% dextrose medium, the same phenomenon of primary acidity and secondary alkalinity would be observed.

The Care of Stock Cultures.—Stock cultures of the gonococcus can be kept best by daily transfers on testicular agar: If transfers are made at less frequent intervals the growths are uncertain and less abundant.

A moist atmosphere, a temperature of 37.5 C., and the proper reaction of the mediums are necessary. A poorly growing culture, if changed to another favorable culture medium, begins to grow more abundantly. The addition of serum to the surface of an agar slant will also improve the growth.

The Addition of Dyes to Culture Mediums.—Studies of the effect of dyes on the growth of the gonococcus and contaminating organisms, have been made. The reaction of the medium and the quantity of nutrient substances, such as glucose and peptone, influence the effectiveness of dyes. We used testicular agar of P_H 7.4 and a 24-hour culture of gonococci and staphylococci.

TABLE 3
THE EFFECT OF VIOLET DYES ON THE GROWTH OF STAPHYLOCOCCI

Staphylococci Different Strains	Aniline Violet		Crystal Violet		Gentian Violet		Methyl Violet	
	Growth	No Growth	Growth	No Growth	Growth	No Growth	Growth	No Growth
Stock culture.....	1:25,000	1:10,000	1:25,000	1:10,000	1:50,000	1:40,000	1:40,000	1:25,000
Clinical 1.....	1:10,000	1:5,000	1:75,000	1:50,000	1:50,000	1:40,000	1:5,000	1:2,500
Clinical 2.....	1:40,000	1:25,000	1:75,000	1:50,000	1:10,000	1:5,000	1:10,000	1:5,000
Clinical 3.....	1:2,500	1:1,000	1:5,000	1:1,250	1:10,000	1:5,000	1:5,000	1:2,500
Clinical 4.....	1:50,000	1:40,000	1:10,000	1:5,000	1:75,000	1:50,000	1:40,000	1:25,000
Clinical 5.....	1:40,000	1:25,000	1:140,000	1:120,000	1:60,000	1:50,000	1:60,000	1:50,000
Clinical 6.....	1:60,000	1:50,000	1:140,000	1:120,000	1:60,000	1:50,000	1:60,000	1:50,000
Clinical 7.....	1:40,000	1:25,000	1:120,000	1:100,000	1:25,000	1:20,000	1:60,000	1:50,000
Clinical 8.....	1:50,000	1:40,000	1:180,000	1:160,000	1:100,000	1:75,000	1:75,000	1:60,000
Clinical 9.....	1:60,000	1:50,000	1:20,000	1:10,000	1:10,000	1:50,000	1:40,000	1:25,000
Clinical 10.....	1:60,000	1:50,000	1:20,000	1:10,000	1:10,000	1:5,000	1:40,000	1:25,000

Staphylococci and other gram-positive contaminants²³ can be inhibited to a marked degree by violet and green dyes. Staphylococci vary considerably in their ability to grow in the presence of violet dyes, as shown in table 3. There is also some difference in the bactericidal effect of a given dye made by different firms.²⁴ Aniline, crystal, gentian and methyl violet, and aniline, brilliant, malachite, methyl and solid green were used in our experiments. Methyl violet was found to be the most effective one for the isolation of the gonococcus in cases of mixed infection.

The comparative effect of violet dyes on gonococci and staphylococci is shown in table 4.

²³ Churchman, J. W., and Michael, W. H.: Jour. Exper. Med., 1912, 16, p. 822.

²⁴ Churchman, J. W., and Herz, L. F.: Ibid., 1913, 18, p. 579.

TABLE 4
THE COMPARATIVE EFFECT OF VIOLET DYES ON GONOCOCCI AND STAPHYLOCOCCI

No. of Cultures	Methyl Violet	Methyl Violet	Aniline Violet
Gonococcus.....	1:100,000	1:500,000	1:100,000
Strain 1.....	+++	+++	++
Strain 2.....	+++	+++	++
Strain 3.....	++	+++	++
Strain 4.....	++	++	++
Staphylococcus			
Strain 1.....	+	+	++
Strain 2.....	+	+	++
Strain 3.....	+	+	++
Strain 4.....	++	+	++

+ = Slight growth. ++ = Fair growth. +++ = Good growth.

Combinations of green and violet dyes have also been tried; a concentration of 1:500,000 of each dye (as methyl green and methyl violet) is approximately as effective as a greater concentration—1:200,000—of a single dye.

The dye mediums are prepared by adding a sufficient quantity of a 1:1,000 stock solution of the dye to a measured amount of culture medium. The stock solution of dye is prepared by weighing the dye on tinfoil, and washing it into a sterile flask with sterile distilled water. Neutral glassware must be used and evaporation guarded against.

Victoria blue, janus green, hydroquinone, quinone, pyronine, magenta red, congo red and pyoktanin blue vary in their inhibitory action. None of them are of value in inhibiting contaminating organisms in a mixed culture. Hexamethylenamine was added to testicular agar of different reactions. It does not inhibit the growth of staphylococci or colon bacilli as much as that of the gonococcus. The practical importance of violet dyes, especially methyl in 1:200,000 to 1:500,000 concentration for isolation purposes was demonstrated in acute cases. In approximately 40% of acute cases we were able to obtain a growth of the gonococcus on methyl violet tubes, with marked inhibition of the staphylococcus, as indicated by comparison with tubes which contained no violet dyes. This is best demonstrated by the growth of transplants from the violet tubes on blood testicular agar. By this method the isolation of the gonococcus from acute and subacute cases of vaginitis of children was simplified. It is difficult to isolate the gonococcus from a chronic infection of the female genital tract. Nevertheless, because of the great practical importance of making a definite diagnosis, it is well worth while to attempt the isolation.

In transferring exudates²⁵ to plates or tubes, dry cotton swabs are not reliable; the swab should first be moistened in the water of condensation of a tube of culture medium or a platinum wire should be used.

SUMMARY AND CONCLUSIONS

Testicular blood agar with a reaction of P_H 7.4-7.8 is the most favorable medium for the isolation and subsequent cultivation of the gonococcus. The medium is prepared thus:

Beef testicle from which all connective tissue has been removed is put through a meat grinder, weighed and with twice its weight of distilled water added, is infused over night on ice. The following morning the mixture is heated in a double boiler to 50 C., allowed to stand for one hour and then brought to the boiling point. Let it stand for another hour to permit the solid particles to settle, after which the liquor is decanted off and used as the infusion for the preparation of culture mediums.

For the preparation of testicular infusion agar, 2% peptone, 0.5% glucose, 0.2 to 0.3% monobasic sodium phosphate and 2.5% granular agar are added. This is heated over a flame and stirred constantly until the agar is dissolved. The medium is titrated with phenol red as an indicator and the reaction adjusted to P_H 7.4 to 7.8; if phenolphthalein is used as an indicator, it should be adjusted to a 0.6 reaction. The medium is tubed and autoclaved for 20 minutes at 15 lbs. The titration is checked after sterilization. While the tubes are still liquid (just before the agar solidifies) human blood in the proportion of 0.5 to 2.5% is added. If human blood is not available, defibrinated rabbit's blood (1-5%) may be substituted.

The absence of sodium chloride, the proper reaction and moisture content are especially important.

Blood or blood serum mixed with the testicular agar or smeared on the surface of slanted tubes is necessary for the ready isolation of the gonococcus, but is not essential for the securing of growths of stock cultures.

Fermentation tests made with solid mediums containing 0.5% glucose and phenol red as an indicator results in a significant primary acidity and secondary alkalinity.

²⁵ Van Gieson, Ira: N. Y. Med. Rec., 1910, p. 1001.

Reduced oxygen tension is of no practical value for either the isolation or subsequent cultivation of the gonococcus.

Aniline dyes of the violet and green colors tend to inhibit the growth of staphylococci more than that of gonococci from cases of mixed infection. Methyl violet added to blood-testicular agar in a proportion of 1:200,000 to 500,000 appears to be of greatest value.